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"Reversal of Acetylcholinesterase Inhibitor Toxicity
in vivo by Inhibitors of Choline Transport"

Final Report

John J. Freeman, Ph.D.
Joseph W. Kosh, Ph.D.

October 31, 1983

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The antagonism of acetylcholinesterase inhibitor toxicity was studied in mice. Hemicholinium-3 (HC-3) antagonized the toxicity of physostigmine and neostigmine by shifting the dose-response curve to the right, thus increasing the LD50. However, hemicholinium-3 failed to have a protective effect against disopropylfluorophosphate (DFP), causing an enhancement of the toxicity. Hemicholinium-3 shifted the dose-response curve to the left of DFP's. Neostigmine, a quaternary nitrogen compound, increased the level of acetylcholine and choline in the brain. Since

20. (Continued)

physostigmine also caused a similar response, the results indicated that neostigmine also crossed the blood-brain barrier. This was confirmed when neostigmine was shown to inhibit brain acetylcholinesterase following systemic administration.

Pretreatment, concurrent treatment and postreatment with hemicholinium-3 (HC-3) did not consistently decrease the effects of physostigmine and neostigmine on acetylcholine levels in either the heart or brain. Thus, hemicholinium-3 may be exerting a different effect. The protecting effect of hemicholinium-3 on acetylcholinesterase inhibitor toxicity may be due to blockade of the post-synaptic receptor at the neuromuscular junction. The discrepancy between the effect of hemicholinium-3 on the reversible and irreversible inhibitors remains unexplained.

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"The findings in this report are not to be construed as an official
Department of the Army position unless so designated by other
authorized documents."

(ii)

Forward

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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INTRODUCTION

Pharmacology of Acetylcholinesterase Inhibitors

The function of acetylcholinesterase (AChE) is to terminate the action of

acetylcholine (ACh) at the cholinergic receptor. True acetylcholinesterase is

located adjacent to the receptor while pseudocholinesterase is found in the

blood. Stewart (1) and Douglas and Paton (2) suggested that the

physiological effects of cholinesterase inhibitors are due to the increased

interaction of acetylcholine with the receptor resulting from the inhibition

of the enzyme acetylcholinesterase.

Acetylcholinesterase inhibitors can be divided into three categories depending

on their duration of action and how they work. "True" cholinesterase inhibitors

are competitive inhibitors of acetylcholine at the enzyme receptor. The second

category, "reversible" cholinesterase inhibitors, form covalent bonds with the

receptor in a reversible manner. The third category, "irreversible"

cholinesterase inhibitors, also form covalent bonds with the receptor, but the

bond that is formed is essentially irreversible.

The reversible cholinesterase inhibitors, physostigmine and neostigmine, have

also been shown to inhibit the synthesis of ACh in guinea pig brain (3) in

addition to their acetylcholinesterase inhibiting effect. Physostigmine but

not neostigmine, has been shown to decrease the amount of ACh released from

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the cholinergic nerve terminal following nerve stimulation (4). Neostigmine
has also been shown to have a direct effect on the nicotinic receptor of mammalian
neuromuscular junctions. Diisopropylfluorophosphate (DFP) is an example of an
irreversible organophosphorous cholinesterase inhibitor. DFP has also been shown
to produce an increase in the total acetylcholine content in the brain of the rat
and rabbit.

The toxicity of cholinesterase inhibitors (whether reversible or irreversible)
is due to excessive accumulation of acetylcholine which causes continous
stimulation of the post-synaptic receptor (5,6). Thus the receptor can be
stimulated to the point where it can no longer respond resulting in paralysis.
The cause of death is anoxia due to a combination of factors:

    1) *Central and peripheral respiratory paralysis, and*

    2) Severe bronchoconstriction complicated by excessive bronchial and

      salivary secretions.

The signs and symptoms of acute poisoning by anticholinesterase compounds have
been reported to be the same whether the toxic substance is an irreversible or
a reversible inhibitor of cholinesterase. The same symptomology has been reported
for poisoning by physostigmine, neostigmine or irreversible compounds such as
DFP. (7,8). The most important difference between reversible and irreversible
cholinesterase inhibitors is that the equilibrium conditions are attained rapidly
with the reversible inhibitors while inhibition produced by the irreversible
inhibitor is slower to develop.

Cholinesterase inhibitors apparently have two mechanisms of action. At lower concentrations only inhibition of cholinesterase is seen, but at higher concentrations, a "curare-like" blockade is observed (9). Inhibition of acetylcholinesterase at myoneural junctions is more closely correlated with toxicity (10). Pharmacological observations have shown that the signs of poisoning produced by many inhibitors can be explained without postulating any direct interference with the central nervous system. Inhibition and recovery of brain acetylcholinesterase are not related to toxicity in any simple way.

General Pharmacology of Hemicholinium-3 (HC-3)

The hemicholiniums were first synthesized by Long and Schueler (11) in 1954. Chemically, the hemicholiniums are characterized by a choline moiety cyclized through hemiacetal formation. Pharmacologically, their most striking characteristic is their ability to cause respiratory failure (12): Schueler (13) first demonstrated that hemicholinium-3 (HC-3) produced a neuromuscular blockade in several skeletal muscle preparations.

The most likely mechanism of action of HC-3 includes a presynaptic as well as a postsynaptic action. A presynaptic action for HC-3 was first postulated by MacIntosh et al. (14) and later confirmed by Gardiner (15). Gardiner showed ACh synthesis was inhibited in intact but not in disruped synaptosomes. Other

investigators have since confirmed that HC-3 blocks choline uptake into synaptosomes
(16-18). Hemicholinium-3 has been shown to mainly inhibit the high affinity uptake
system for choline in a competitive manner (19). The high affinity system is required
for the synthesis of ACh (20-22).

Other evidence for a presynaptic effect of hemicholinium-3 has been provided
by nerve stimulation studies and by choline uptake studies. In general, the
higher the rate of nerve stimulation, the more effective hemicholinium-3 is in
blocking cholinergic nerve transmission (23,12). The specific antagonism of HC-3
by choline suggested that HC-3 was interfering with choline transport into the
nerve terminal (24). Furthermore, choline has been observed to antidote the toxic
action of HC-3 at autonomic neuroeffector junctions, sympathetic ganglia, brain
tissue and at neuromuscular junctions in anesthetized mice, rats, rabbits, cats,
dogs and chickens (13, 23, 25).

Hemicholinium-3 has been shown to cause a decrease in the synthesis of acetylcholine
by blocking the uptake of choline into the nerve terminal. This results in a
decrease in the turnover of acetylcholine (26), as well as a decrease in the level
of acetylcholine.

The site of HC-3 toxicity has always been controversial. Most investigators have
suggested a peripheral site of action but several have concluded that the drug
exerts its toxic action centrally. A peripheral site of toxicity for HC-3 was
consistent with the well-known inability of quaternary ammonium compounds to
pass through the blood-brain barrier.

A central site of action was first suggested by Schueler (13). Using a cross circulation experiment in anesthetized dogs, Schueler concluded that HC-3 was acting in the spinal cord to depress respiration. Kase and Borison (27) concluded that HC-3 depressed respiration centrally after stimulating the respiratory center in cats at the level of the brainstem. Using several centrally acting depressant drugs, Clement (28) concluded that HC-3 was also active centrally. Clement suggested that the anesthetic might be interfering with the central action of HC-3 thus protecting the animal against the central toxic effects of HC-3.

Studies which looked at the distribution of HC-3 after an i.p. dose have shown HC-3 does not readily penetrate the blood-brain barrier (29-31). Evidence by Schueler (13) substantiated the suggestion that HC-3 did not cross the blood-brain barrier into the brain since no alteration of the electroencephalogram was seen after an intraperitoneal injection. Longo (32) concluded that HC-3 toxicity was peripheral in nature since the effect of lethal doses of HC-3 on efferent phrenic nerve potentials were uneffected in spite of a failure of respiration. Holmes and Wilson (33) also concluded that HC-3 possessed a peripheral site of action. More recently, Freeman et al. (34) have demonstrated that hemicholinium-3 exerted its toxicity in the periphery following intraventricular administration.

Combination of Acetylcholinesterase Inhibitors and Hemicholinium-3

Atropine and oximes comprise the standard treatment for cholinesterase

toxicity. However, treatment with atropine and an oxime is ineffective in

animals poisoned by the organophosphorous cholinesterase inhibitor soman

(35). This is due to the soman inhibited cholinesterase rapidly aging to

a form which is resistant to oxime reactivation (36). Furthermore, atropine

is ineffective in reversing the paralysis due to blockade of peripheral

neuromuscular transmission which is the cause of the respiratory paralysis.

For prophylaxis against aging, as well as treatment of toxicity, more

fruitful approaches would be appropriate (37).

The most important cause of the acute toxicity of cholinesterase compounds

is accumulation of acetylcholine subsequent to inhibition of cholinesterase.

The acetylcholine which acts locally on various receptors originates largely

by release from nerve endings (5, 6, 38). A reduction in the receptor

interaction of acetylcholine should reduce the toxicity. A reduction could

be accomplished by three mechanisms:

1) Increase the activity of acetylcholinesterase

2) Block the receptor

3) Reduce the release of acetylcholine

Hemicholinium-3 is a well established inhibitor of choline transport in vitro. An inhibition of high affinity choline uptake at the cholinergic nerve terminal also appears to provide a satisfactory explanation of its effects in vivo (39). Therefore, in spite of the evidence for a postsynaptic action of HC-3, the predominant effect of HC-3 appears to be presynaptic.

Recent studies which have investigated the interaction between cholinesterase inhibitors and HC-3 have reversed the blockade of neuromuscular transmission produced by HC-3 with non-lethal doses of a cholinesterase inhibitor (40). Hemicholinium-3 was therefore used as a model for myasthenia gravis and cholinesterase inhibitors were used to reverse the muscle weakness.

The notion that inhibitors of choline uptake might be useful for treating cholinesterase toxicity is not new. Wills in 1970 (41) stated:

> "Of the compounds that interfere with the production of
> acetylcholine within nerve cells, two that have been studied
> as possible adjuncts to atropine are the flavonoid morin and
> hemicholinium-3. Despite the fact that hemicholinium-3 has
> been found to lower concentrations of ACh in such tissues as
> auricle, intestine, iris and ciliary body, but not skeletal
> muscle, even those stimulated this compound has not been found
> to be effective therapeutically in cats poisoned with sarin (42)."

A search of the Wills article of 1963 (42) revealed that while in vivo neuro-
muscular studies were suggestive of a beneficial response, in vitro toxicity
studies were not determined.

It is the purpose of this report to investigate mechanism number 3 above:
the effect of reducing the release of acetylcholine on the toxicity of
acetylcholinesterase inhibitors.

The hypothesis of the proposed study is that treatment of rats or mice with
inhibitors of cholinergic transmission should decrease the toxicity of
cholinesterase inhibitors. The rationale is that any compound which decreases
the release of acetylcholine will decrease the interaction of acetylcholine
with the receptor and decrease the toxicity of cholinesterase inhibitors.

A related hypothesis is that inhibitors of choline transport or inhibitors
of cholinergic neurotransmission will retard the increase in acetylcholine
levels caused by cholinesterase inhibitors. In some instances, the inhibitor
of cholinergic neurotransmission should produce a reduction in the synthesis
which should be detectable by a decrease in the level of acetylcholine. A
reduction in the release of acetylcholine should correlate with a reduction
in the toxicity of cholinesterase inhibitors.

METHODS

Animals

Male HA/ICR mice weighing between 25 and 35 grams were used in this study. Mice were supplied by Harlan Sprague Dawley Industries, Indianapolis, Indiana.

Toxicity Determination of Physostigmine and Neostigmine

Groups of ten mice were administered physostigmine intraperitoneally in eight doses ranging from 0.5 mg/kg to 1.2 mg/kg. Neostigmine was given in nine doses ranging from 0.3 to 1.1 mg/kg. Diisopropylfluorophosphate (DFP) was given in the dose range of 9.0 to 10.8 mg/kg. The number of mice killed in each group and time to death of each mouse were recorded.

Effect of Hemicholinium-3 (HC-3) on the Toxicity of Physostigmine, Neostigmine, and Diisopropylfluorophosphate

Nonlethal doses of hemicholinium-3 (HC-3) were administered to mice intraperitoneally in the schedule listed below:

1) HC-3 pretreatment + physostigmine (or neostigmine or DFP): HC-3 was given 30 minutes before physostigmine (or neostigmine or DFP).

2) HC-3 + physostigmine (or neostigmine or DFP) HC-3 was given

    simultaneously with physostigmine (or neostigmine).

3) Physostigmine (or Neostigmine or DFP + HC-3 postreatment: HC-3

    was given two minutes following physostigmine (or neostigmine).

In each treatment, percent of mice killed in each group and the time to death
of each mouse were recorded. A "Least squares curve fit" program was used to
draw the sigmoid curve of the log dose-response curves.

Probit Analysis

The percent of mice killed were transformed into a probit number according to
the table of "Probit Transformation" found in the Science Tables (Documentia,
Geigy) according to the method of Riggs (43). The log dose and probit number
were also evaluated with the "Least squares curve fit" program to obtain a linear
relationship.

Measurement of Acetylcholine (ACh) and Choline (Ch) in Mouse Brain and Heart

The gas chromatographic assay of Kosh et al. (44) was used to determine the
level of ACh and Ch in brain and heart tissue. Mice were sacrificed with whole
body microwave irradiation (2.0-2.4 seconds, full power). Hearts were washed
with distilled water after removal from the body. Brains and hearts were then
weighed and homogenized with a Polytron (Brinkman Instruments) in two ml of 15%
1N formic acid - 85% acetonitrile containing 5 nmoles butyrylcholine (BCh) as the
internal standard. The sample was carried through the assay as previously
described (44).

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Two ul of the chloroform layer was injected into a Hewlet Packard 5880 gas chromatograph equipped with a nitrogen phosphorus detector (NPD). Area ratios (ACh/BCh and Ch/BCh) were obtained by dividing the ACh and Ch area by the BCh area.

Measurement of Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart Treated with Physostigmine (or Neostigmine or DFP) and Hemicholinium-3 (HC-3) Intraperitoneally

The measurement of ACh and Ch levels in mouse brain and heart were performed with physostigmine alone, HC-3 pretreatment + physostigmine, HC-3 + physostigmine concurrently or physostigmine + HC-3 postreatment with the LD50 doses of physostigmine of 0.69, 0.72, 0.83 and 0.85 mg/kg respectively. (The LD50 doses of neostigmine when given alone, with HC-3 pretreatment, with HC-3 concurrent treatment and with HC-3 postreatment were 0.55, 0.72, 0.70 and 0.80 mg/kg respectively). DFP was given in a dose range from 6 to 11.5 mg/kg. Mice were microwaved upon death or at the maximum time to death. Brain and heart were homogenized in two ml 15% 1N formic acid-85% acetonitrile and assayed according to the method described above.

Measurement of Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart Treated with Hemicholinium-3 (HC-3) Alone Intraperitoneally

The ACh and Ch levels in mouse brain and heart were measured in mice treated with 25 μg/kg HC-3 for 10, 20, 30 or 40 minutes. Mice were microwaved and brains and hearts were removed and assayed as above.

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Protein Determination in Mouse Brain

The method of Bradford (45) was used to measure protein. Briefly, the assay
involves the binding of Coomassie Brilliant Blue G-250 to protein which produces
an absorbance at 595 nm.

Determination of Acetylcholinesterase Activity in Mouse Brain

The specrotphotometric method of Ellman et al. (46) was used to determine the
acetylcholinesterase activity in mouse brain homogenates. Briefly, the enzyme
activity was measured by following the increase in absorbance produced from thio-
choline as it reacts with the dithiobisnitrobenzoate ion. The reaction rates
were recorded with a Gilford spectrophotometer and recorder.

Determination of the Effect of Neostigmine (ip) on the Cholinesterase Activity
in Mouse Brain

Mice were administered the LD50 dose (0.55 mg/kg) of neostigmine intraperitoneally.
The brain was removed and homogenized in 0.25 ml phosphate buffer (pH 7.9)
following their death. Surviving mice were sacrificed 780 seconds after the
injection and the brain tissue then homogenized. The homogenate was centrifuged
at 15,000 RPM for twenty minutes. Cholinesterase activity was determined according
to the method described above. The amount of protein in the mouse brain super-
natant was calculated according to the standard linear equation obtained from
the assay of bovine serum albumin.

RESULTS

The Effect of Hemicholinium-3 (HC-3) on Physostigmine Toxicity

Figure 1 illustrates the log dose-response curve of physostigmine in mice
and the effect of HC-3 on the curves. The curve shifted to the right and
the LD50 of physostigmine was increased by administration of HC-3. The
figure demonstrates that pretreatment, concurrent treatment and postreatment
with HC-3 antagonized the toxicity of physostigmine. Concurrent treatment of
physostigmine and HC-3 had the most consistent effect of shifting the dose-
response curve to the right. Two minutes postreatment with HC-3 also had a
pronounced effect on the dose-response curve by shifting it to the right.

Figure 2 is the probit analysis of the effect of HC-3 on physostigmine treatment
in mice. By examining the probit value of 5, the LD50 of physostigmine with
various treatments of HC-3 was obtained. The LD50 of physostigmine treatment
alone was 0.69 mg/kg. The LD50 was increased to 0.72, 0.83 and 0.85 mg/kg m
pretreatment, concurrent treatment and postreatment respectively with HC-3.

Effect of HC-3 on the Time to Death of Mice Treated with Physostigmine

In addition to the lethal dose response (Fig. 1 and 2), the average time to
death was also recorded (Table 5). Mice which were administered physostigmine
usually died within 900 seconds. Behaviorally, death was preceeded by gasping
for approximately one minute followed by tonic-clonic convulsions. Mice which
did not die within 1200 seconds survived without significant problems and did

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not experience tonic-clonic convulsions. The number in parenthesis represents the number of deaths occurring in a group of 10 mice. There appeared to be a negative correlation between percent mortality and time to death. Figure 3 illustrates the linear relationship between the time to death plotted against the dose in mg/kg. Pretreatment and concurrent treatment tended to increase the time to death compared to physostigmine alone, especially at higher doses. The curve representing physostigmine plus HC-3 postreatment is relatively short since the animals either survived or reached their LD100 in a very narrow range.

Effect of Hemicholinium-3 (HC-3) on Neostigmine Toxicity

Figure 4 illustrates the lethal log dose-response curve of neostigmine in mice and the effect of pretreatment, concurrent and postreatment of HC-3 on the curve. Regardless of the treatment schedule, HC-3 shifted the dose-response curve to the right enhancing the LD50. Postreatment of HC-3 was the most effective treatment in shifting the curve to the right, since the LD50 was increased from 0.55 to 0.80 mg/kg.

Figure 5 represents the probit analysis of the effect of HC-3 on neostigmine toxicity in mice. The LD50 of neostigmine treatment alone was 0.55 mg/kg, and was increased to 0.70, 0.72 and 0.85 with concurrent treatment, pretreatment and postreatment respectively.

Effect of Hemicholinium-3 (HC-3) on the Time To Death of Mice Treated with Neostigmine

The average time to death was also recorded for neostigmine when the toxicity curves were determined. Mice which were killed by neostigmine died within 1020

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seconds of the injection (Table 6). Behaviorally, death was preceeded by gasping for approximately one minute followed by tonic clonic convulsions. Animals which survived never exhibited tonic clonic convulsions. The number in parenthesis represents the number of deaths occurring in the group of 10 mice. There was a negative correlation between percent mortality and time to death (data not shown). Fig. 6 illustrates the linear relationship between the time to death and dose. The data illustrates that protection produced by HC-3 pretreatment, concurrent treatment, and postreatment prolongs the time to death. Concurrent treatment with neostigmine and HC-3 prolonged the time to death to over 600 seconds.

The Effect of Hemicholinium-3 (HC-3) on the Lethal Log Dose-Response Curve of Diisopropylfluorophosphate (DFP)

Figure 7 illustrates the log dose response curve of diisopropylfluorophosphate (DFP). The curve was shifted to the left and the LD50 of diisopropylfluorophosphate decreased by pretreatment with HC-3. The figure demonstrates that pretreatment concurrent treatment and postreatment of HC-3 potentiated the toxicity of hemicholinium-3. Postreatment of DFP had the most dramatic effect of shifting the dose-response curve to the left.

Figure 8 is a probit plot of the effect of HC-3 on diisopropylfluorophosphate treatment in mice. The LD50 of diisopropylfluorophosphate (DFP) with various treatments of DFP can be obtained. The LD50 of DFP alone was 10 mg/kg and was decreased to 0.85 mg/kg by postreatment.

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The Effect of Hemicholinium-3 (HC-3) and Physostigmine on the Acetylcholine
(ACh) and Choline (Ch) Concentration of Mouse Brain and Heart

The purpose of this experiment was to determine if the toxicity of physostigmine
was related to the concentration of ACh and Ch in the heart and brain of mice
(Table 1 and 2). The LD50 of physostigmine used for each treatment was taken
from Fig.2. Physostigmine alone caused a significant increase in the amount
of ACh in the brain from 30.4 nmole/g to 43.1 nmole/g. HC-3 pretreatment,
concurrent, and postreatment did not decrease the elevation of ACh or Ch
levels caused by physostigmine in the brain (Table 1).

Table 2 illustrates the effect of HC-3 and physostigmine on the ACh and Ch
concentration of the mouse heart. The LD50 dose of physostigmine did not cause
a significant change in the level of ACh in the heart but it did cause an
increase in the Ch level of the heart. HC-3 pretreatment, postreatment and
concurrent treatment had similar significant effects on increasing Ch, but
not ACh.

The Effect of Hemicholinium-3 and Neostigmine on the Acetylcholine (ACh) and
Choline (Ch) Concentration of the Mouse Brain and Heart

The purpose of this experiment was to determine if the toxicity of neostigmine
is related to the concentration of ACh and Ch in hearts and brains of mice.
The LD50 for neostigmine used for each treatment was taken from Fig. 4.

Neostigmine significantly increased the amount of ACh and Ch in the brain to
41.5 nmole/g and 39.2 nmole/g respectively (Table 3). Pretreatment, concurrent

and postreatment with HC-3 also caused increases in ACh and Ch. Treatment with HC-3 appeared to cause a more pronounced effect on choline than neostigmine alone, although all of the increases in choline were significant (Table 3). Neostigmine also caused a significant increase in the ACh level in the mouse heart (Table 4) but not choline. HC-3 pretreatment, concurrent and postreatment caused variable effects on heart ACh, but caused consistant increases in heart choline. For instance, postreatment with neostigmine increased choline in the heart to 58.5 nmole/g.

Effect of Neostigmine on the Cholinesterase Activity in Mouse Brain Following Systemic Administration

The average rate of cholinesterase activity in control mouse brain was 0.0921 ± 0.009 umole/min/mg. In mice treated with an LD50 of neostigmine (0.55 mg/kg, ip), the average rate of cholinesterase activity decreased significantly to 0.0632 ± 0.005 umole/min/mg. The inhibition of brain acetylcholinesterase activity in neostigmine treated mice showed that neostigmine had a significant effect in the brain following intraperitoneal injection.

Effect of Hemicholinium-3 (HC-3) on the Acetylcholine (ACh) and Choline (Ch) Concentration in Mouse Brain and Heart

The effect of HC-3 alone on the concentration of acetylcholine (ACh) and choline (Ch) in mouse brain and heart is shown in Fig. 9 and Fig. 10. The level of ACh and Ch is expressed as a percent of the control level. Neither the level of ACh nor the level of Ch changed significantly in either the brain or heart compared to control. However, the effect of HC-3 was more pronounced in the heart on both ACh and Ch even though it did not reach significance. No significant decrease

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in Ach levels were observed in the brain and heart tissue following treatment
with HC-3. But the ACh level in the heart tended to decrease. The Ch level
in brain had no significant change either, while the Ch level in the heart
increased but not significantly.

Fig. 1

Plot of the effect of hemicholinium-3 (HC-3) on the lethal log dose-response
of physostigmine. Mice were administered non-lethal doses of HC-3 25 µg/kg
intraperitoneally (ip) 30 minutes before, concurrently, or two minutes after
increasing.doses of physostigmine ranging from 0.5 mg/kg to 1.2 mg/kg. The
percent of mice killed (mortality) at each dose of physostigmine was plotted
versus the log dose. A "Least squares curve fit" program was used to plot the
sigmoid curves. (n = 10/dose)

PHYSOSTIGMINE ALONE
HC-3 PRETREATMENT+PHYSOSTIGMINE
HC-3+PHYSOSTIGMINE CONCURRENTLY
PHYSOSTIGMINE+HC-3 POSTREATMENT

DOSE (mg/kg)

% MORTALITY

Fig. 2

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response

of physostigmine. The percent of mice killed were transformed to a probit

number according to the "probit transformation table". A "Least squares

curve fit" program was used to draw the straight lines. The drug dosage

procedure was the same as in Fig. 1 (n=10/dose).

PROBIT vs DOSE (mg/Kg)

Legend:
— PHYSOSTIGMINE ALONE
--- HC-3 PRETREATMENT+PHYSOSTIGMINE
●●●● HC-3+PHYSOSTIGMINE CONCURRENTLY
---- PHYSOSTIGMINE+HC-3 POSTREATMENT

Fig. 3

Plot of the effect of hemicholinium-3 (HC-3) on the time to death of mice
treated with increasing doses of physostigmine intraperitoneally (ip).
Male mice were administered nonlethal doses of 25 ug/kg HC-3 (ip) 30 minutes
before, concurrently or two minutes after the dose of physostigmine (ip).

Fig. 4

Plot of the effect of hemicholinium-3 (HC-3) on the lethal log dose-response
of neostigmine. Mice were administered non-lethal doses of HC-3 25 ug/kg
intraperitoneally (ip) 30 minutes before, concurrently, or two minutes after
increasing doses of neostigmine ranging from 0.3 to 1.1 mg/kg. The percent
of mice killed (mortality) was plotted versus the log dose of neostigmine.
A "Least squares curve fit" program was used to draw the sigmoid curves.
(n=10/dose)

% MORTALITY

DOSE (mg/kg)

NEOSTIGMINE ALONE

HC-3 PRETREATMENT+NEOSTIGMINE

HC-3+NEOSTIGMINE CONCURRENTLY

NEOSTIGMINE+HC-3 POSTREATMENT

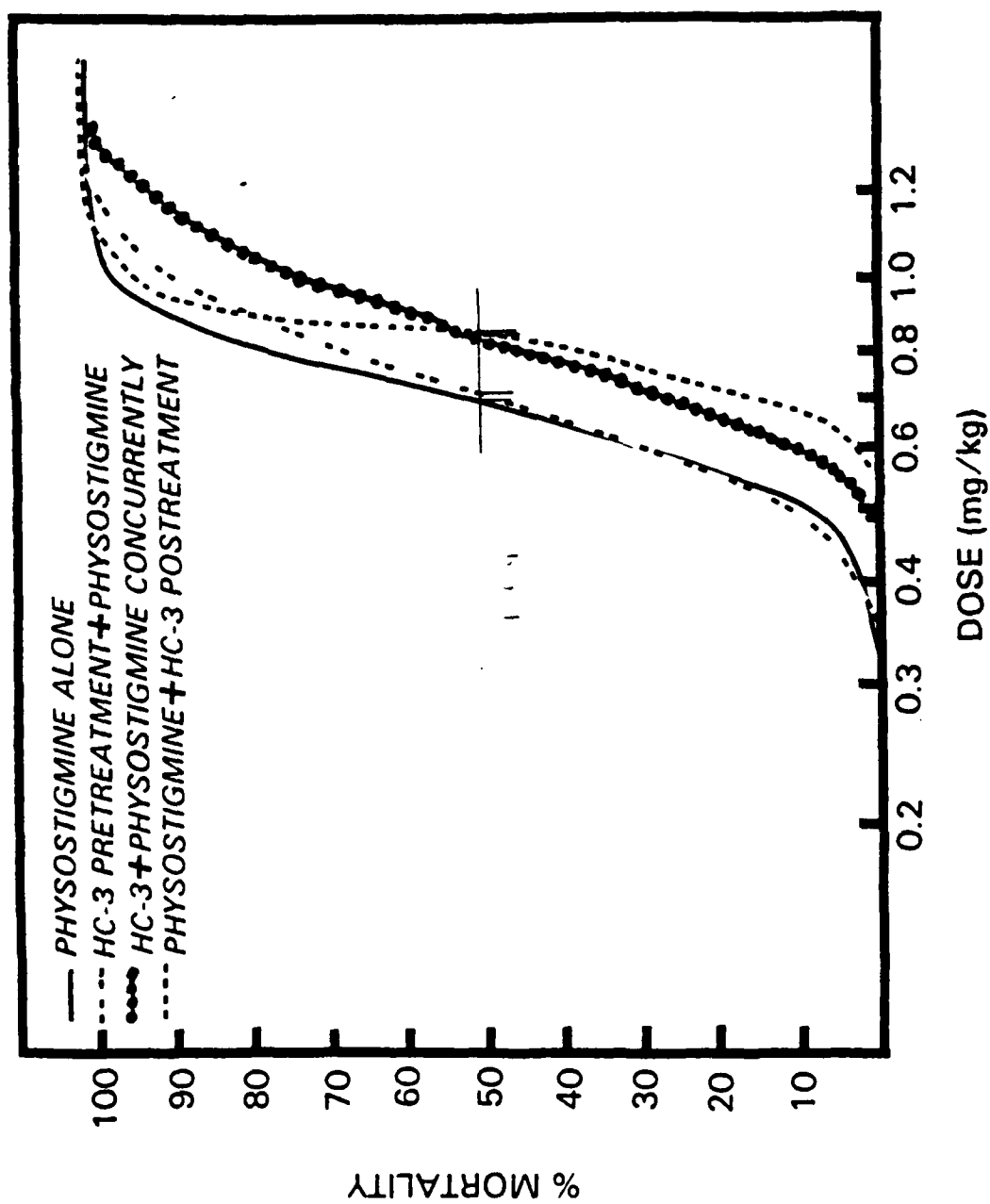100 90 80 70 60 50 40 30 20 10

0.2 0.3 0.4 0.5 0.6 0.8 1.0 1.2

Fig. 5

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response
of neostigmine. The percent of mice killed was transformed into a probit
number according to the "probit transformation table". A "Least squares
curve fit" program was used to draw the straight lines. The drug dosage
schedule was the same as Fig. 4 (n=10/dose).

NEOSTIGMINE ALONE
HC-3 PRETREATMENT+NEOSTIGMINE
HC-3+NEOSTIGMINE CONCURRENTLY
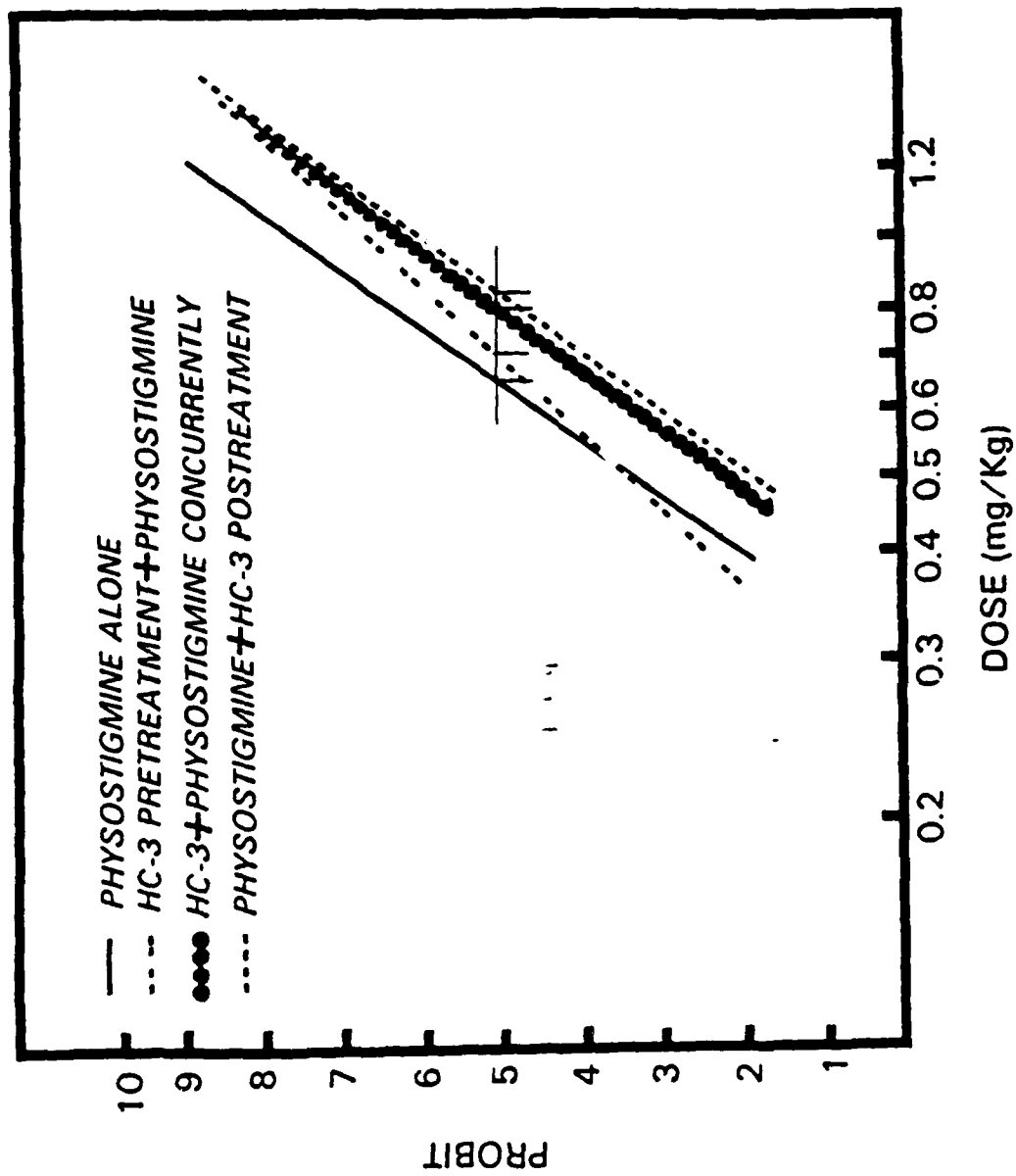NEOSTIGMINE+HC-3 POSTREATMENT

DOSE (mg/Kg)

PROBIT
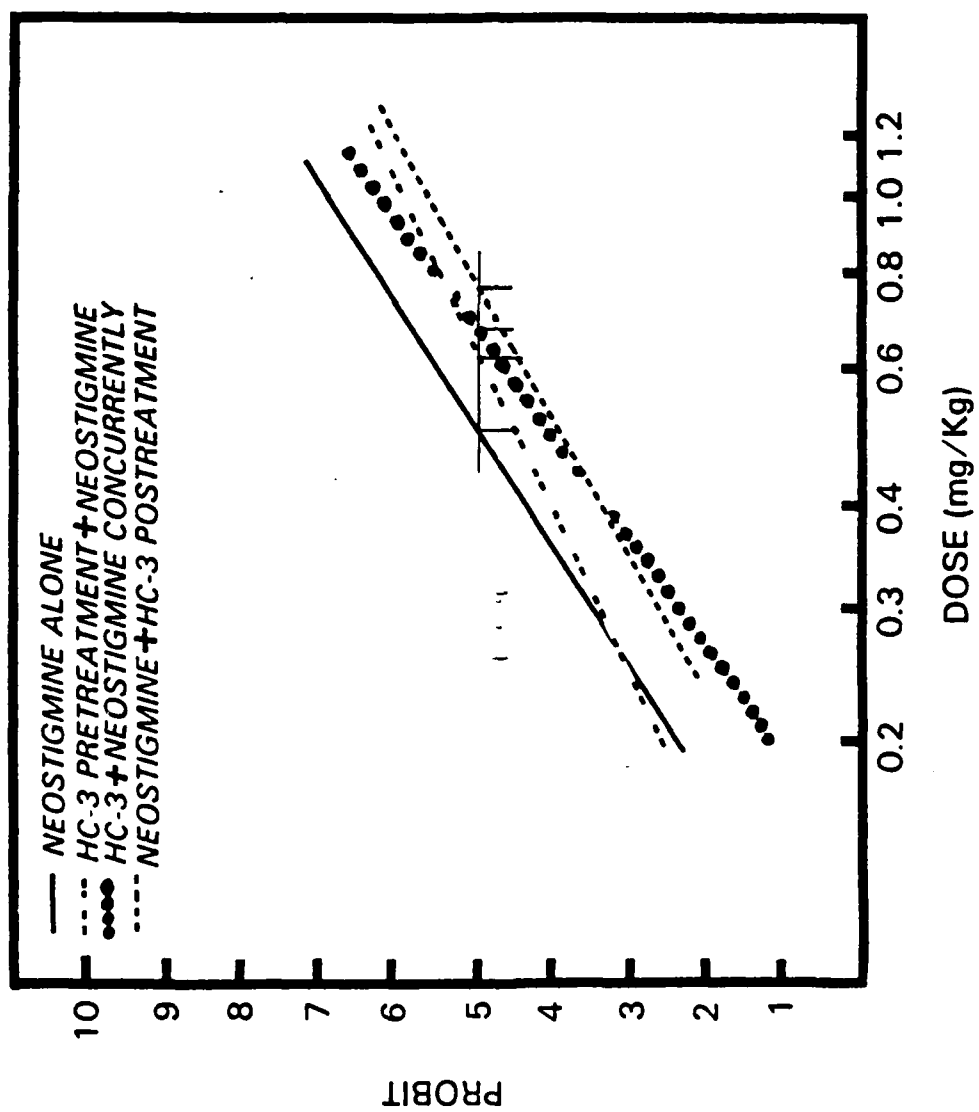
<u>Fig. 6</u>

Plot of the effect of hemicholinium-3 (HC-3) on the time to death of mice
treated with increasing doses of neostigmine intraperitoneally (ip). Male
mice were administered nonlethal doses of HC-3 25 ug/kg (ip) 30 minutes
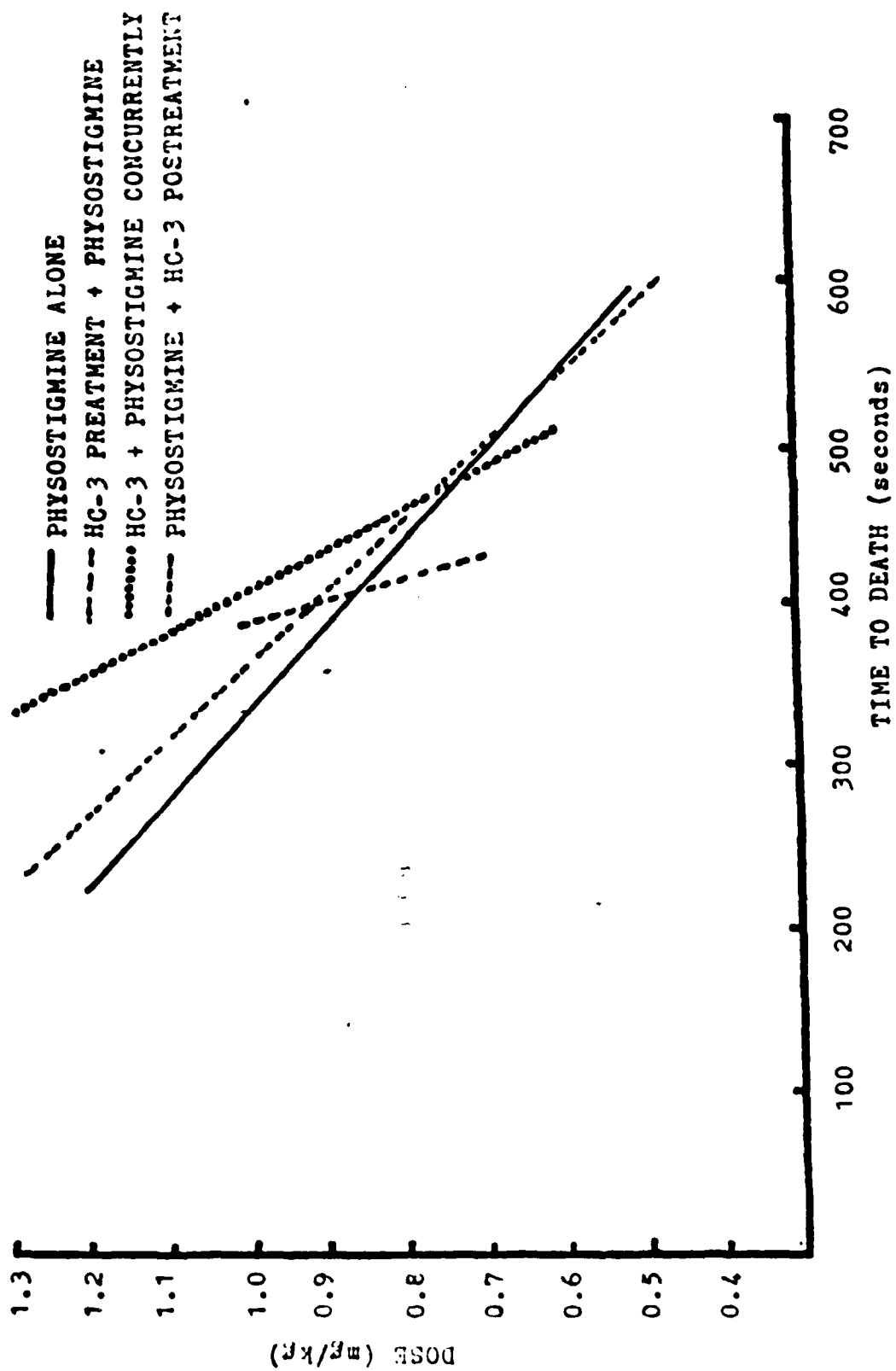before, concurrently or two minutes after the ip treatment with neostigmine.

DOSE (mg/kg)

TIME TO DEATH (seconds)

NEOSTIGMINE ALONE
HC-3 PREATMENT + NEOSTIGMINE
HC-3 + NEOSTIGMINE CONCURRENTLY
NEOSTIGMINE + HC-3 POSTREATMENT
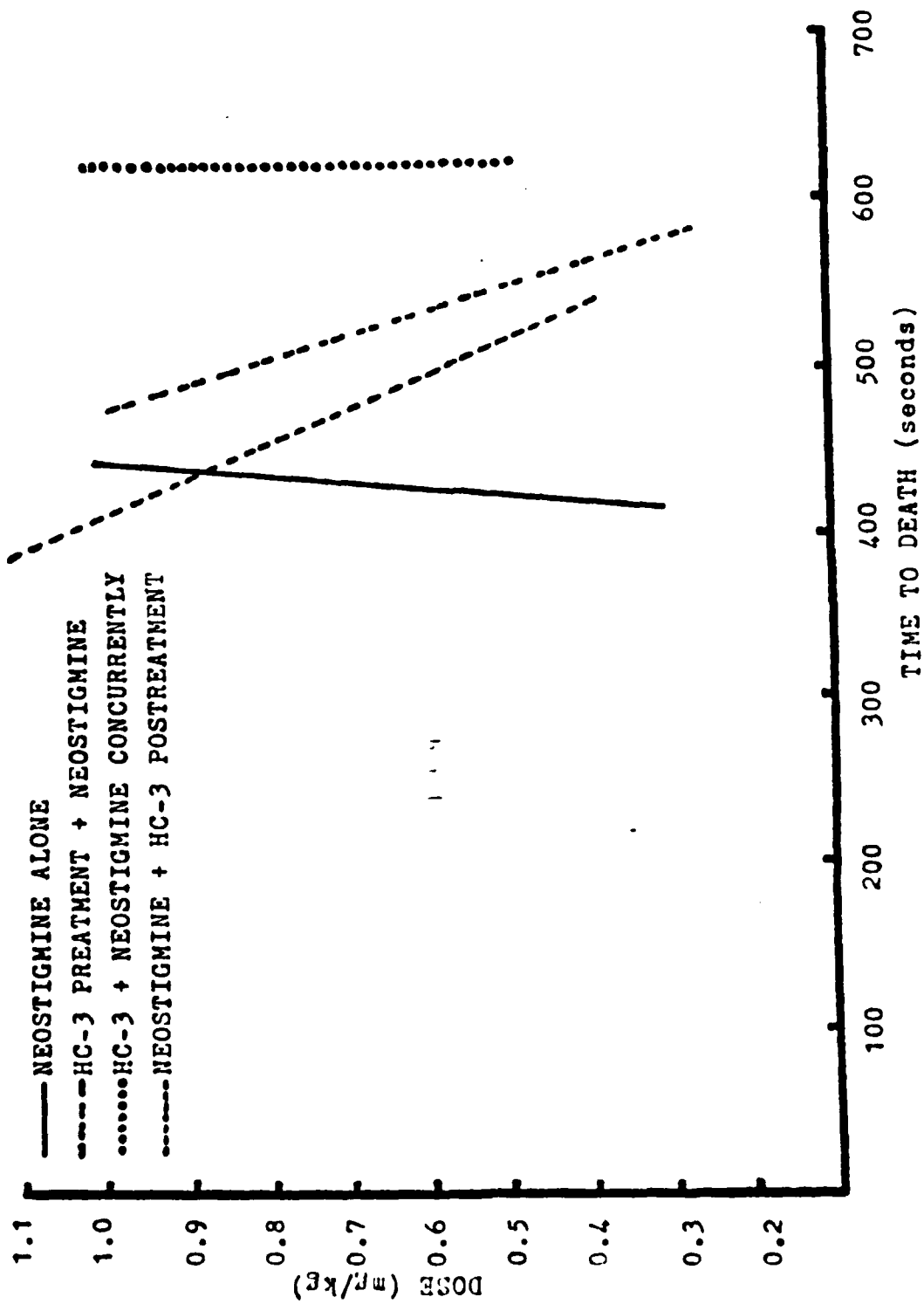
Fig. 7

The effect of hemicholinium-3 (HC-3) on the lethal log dose-response of
diisopropylfluorophosphate (DFP). MIce were administered non-lethal doses
of HC-3 25 µg/kg intraperitoneally (ip) 30 minutes before, concurrently or
two minutes after increasing doses of DFP ranging from 0.6 to 1.2 mg/kg.
The percent mice killed (mortality) at each dose of DFP was plotted versus
the log dose. A "Least squares curve fit" program was used to plot the
sigmoid curves (n=10/dose).

% MORTALITY

LOG DOSE (mg/kg)

— DFP ALONE
-·- HC-3 PRETREATMENT+DFP
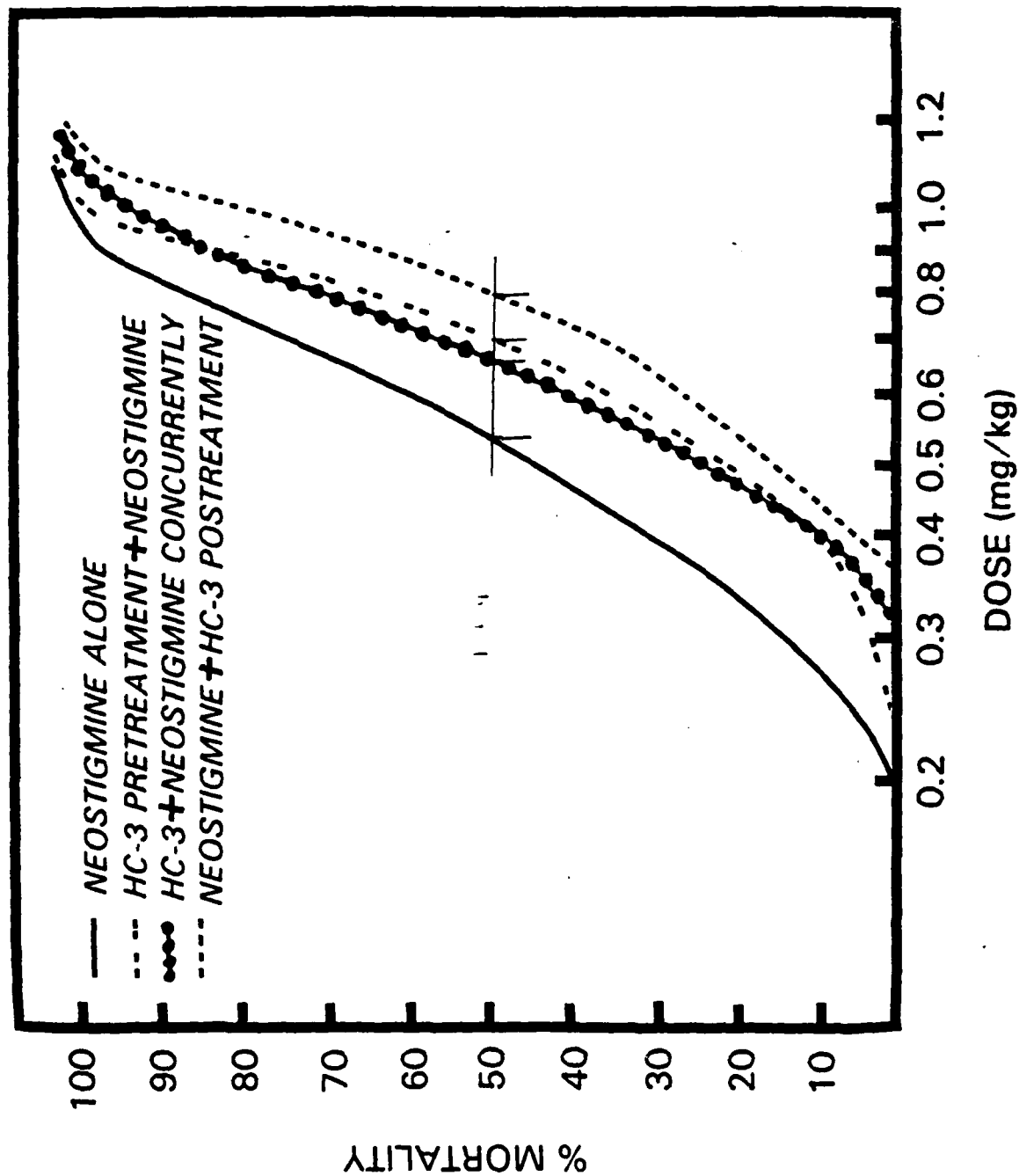●●●● HC-3+DFP CONCURRENTLY
---- DFP+HC-3 POSTREATMENT
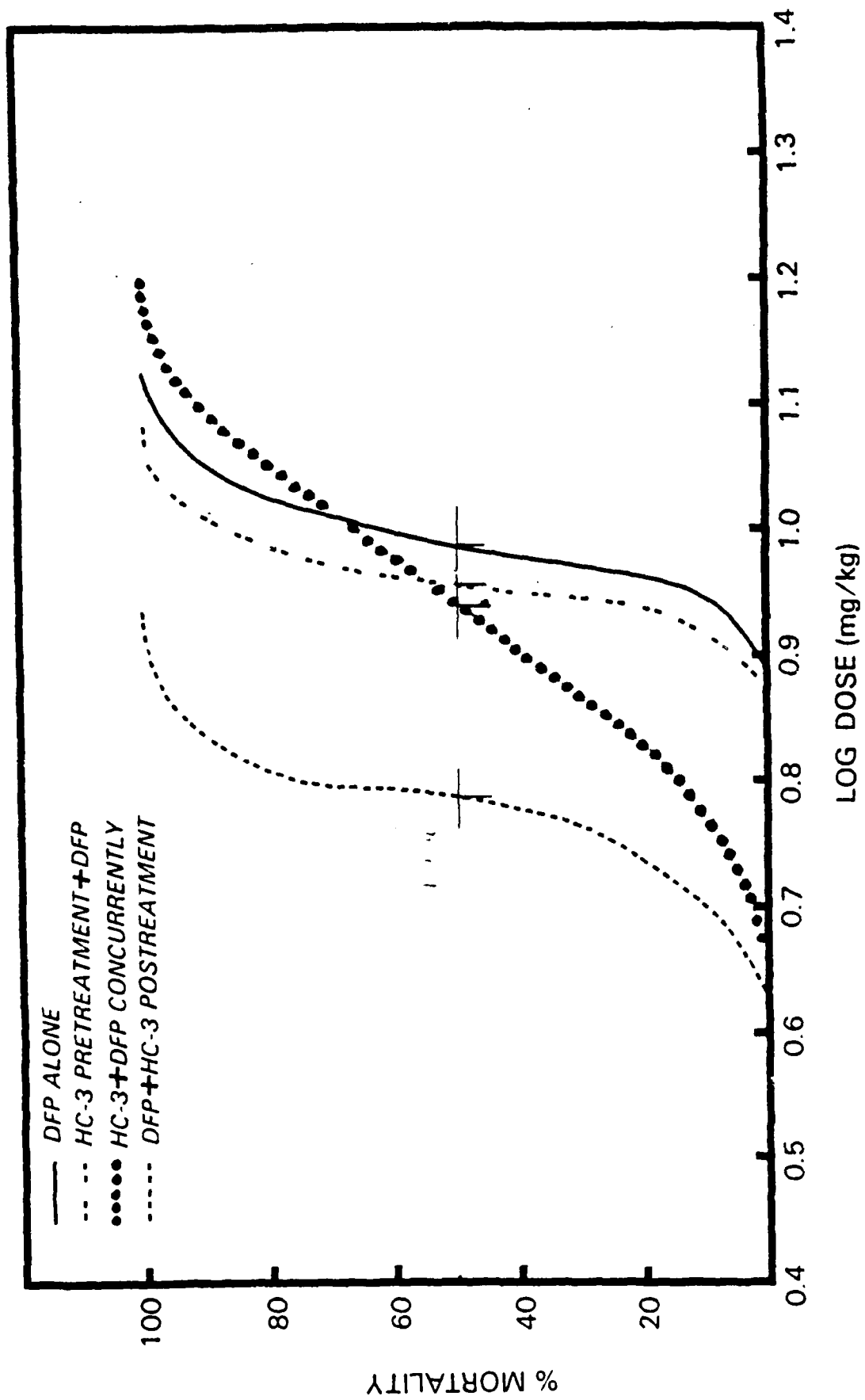
Fig. 8

Probit plot of the effect of hemicholinium-3 (HC-3) on the log dose response
of diisopropylfluorophosphtae. The percent of mice killed was transformed
into a probit number according to the "probit transformation table". A
"Least squares curve fit" program was used to draw the straight lines
(n=10/dose).

PROBIT

LOG DOSE (mg/kg)

DFP ALONE
HC-3 PRETREATMENT+DFP
HC-3+DFP CONCURRENTLY
DFP+HC-3 POSTREATMENT
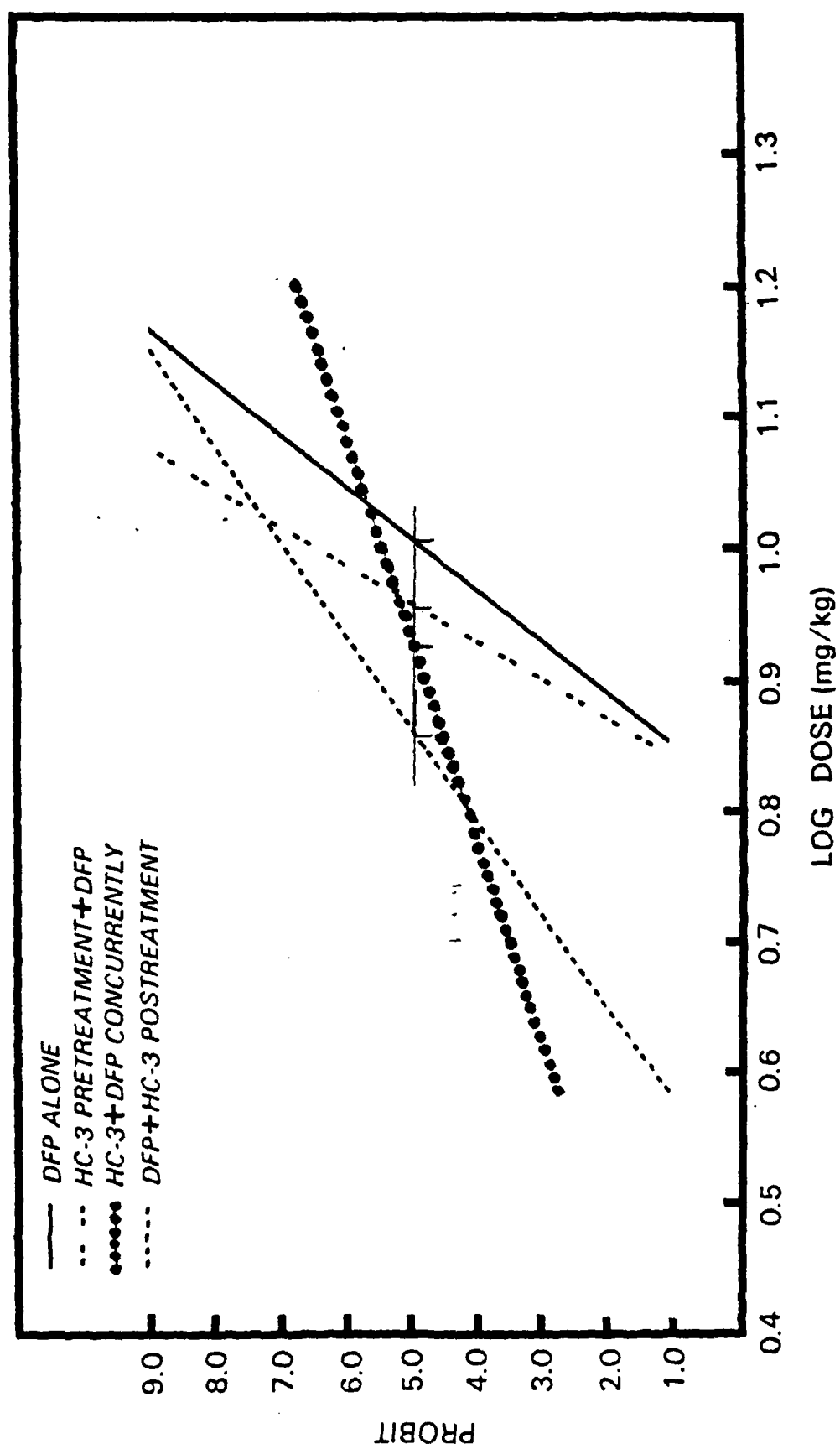
Fig. 9

Effect of hemicholinium-3 (HC-3) on the acetylcholine (ACh) concentration of
mouse brain and heart. Animal whole bodies were microwaved 10, 20, 30 or 40
minutes after the intraperitoneal administration of hemicholinium-3 (HC-3)
25 µg/kg and the brains and hearts analyzed for ACh. Values are expressed as
a percent of control level ± S.E.M. Control levels of ACh in brain and heart
were 30.29 ± 2.40 nmole/g and 3.03 ± 0.45 nmole/g respectively. None of the
values reached significance (n=6).
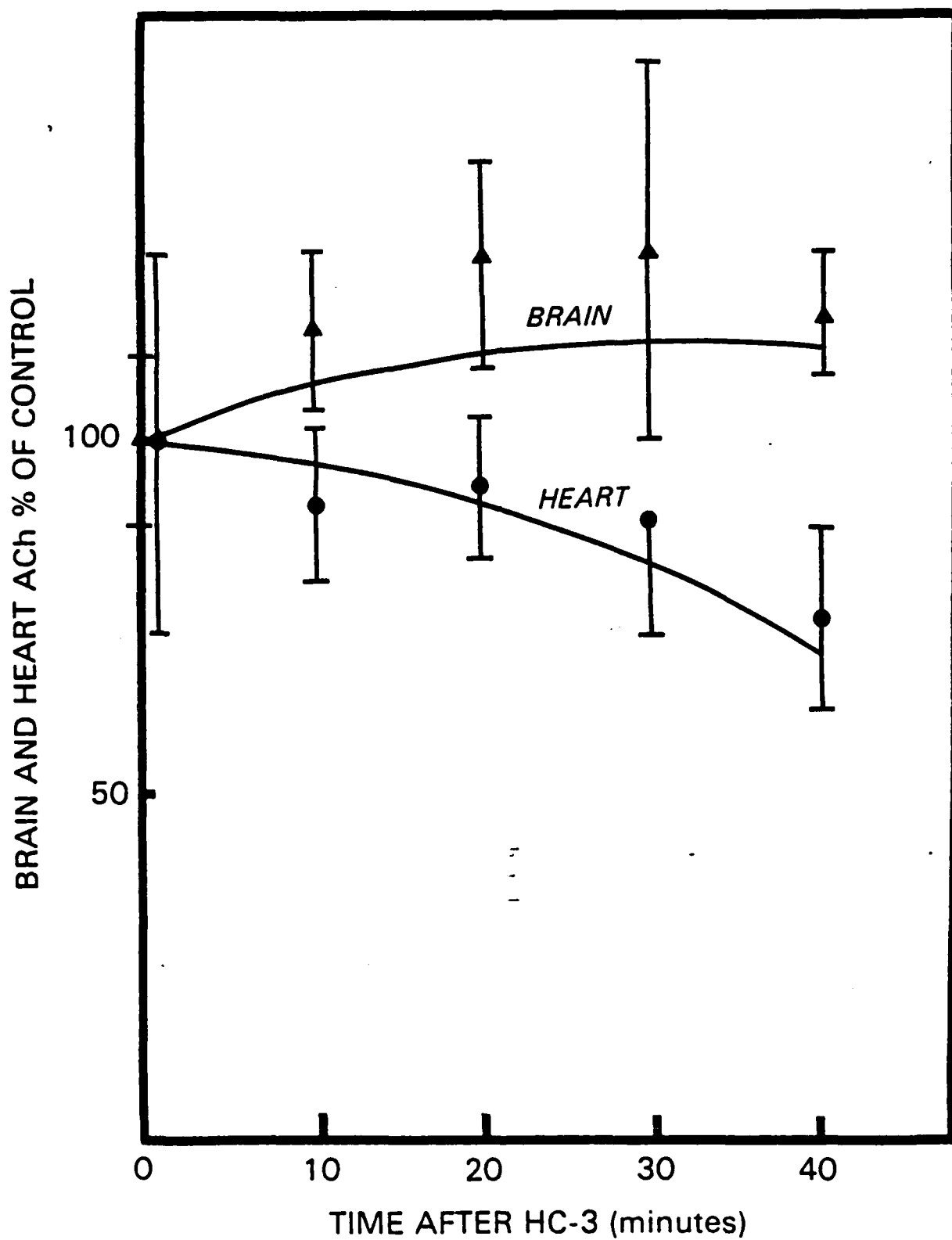
Fig. 10

Effect of hemicholinium-3 (HC-3) on the choline (Ch) concentration of mouse brain and heart. Animal whole bodies were microwaved at 10, 20, 30 or 40 minutes after the intraperitoneal administration of hemicholinium-3, 25 μg/kg and the brains and hearts analyzed for Ch. Values are expressed as a percent of control level ± S.E.M. Control levels of Ch in brain and heart were 22.16 ± 2.45 nmole/g and 38.80 ± 3.53 nmole/g respectively. None of the values reached significance. (n=6).

BRAIN AND HEART Ch % OF CONTROL

HEART

BRAIN

100

50

10   20   30   40

TIME AFTER HC-3 (minutes)

TABLE 1

Effect of Hemicholinium-3 (HC-3)[a] on the Time to Death[b] of Mice

Treated with Physostigmine

| Dose Physostigmine mg/kg	HC-3 Pretreatment Physostigmine	HC-3 Physostigmine Concurrently	Physostigmine HC-3 Postreatment
0.5	660(1)[c]	808(1)	α[d]	α
0.6	535(2)	584(1)	521(1)	α
0.7	693.00±354.93(6)	211.50±185.97(2)	465.00±83.54(4)	380(1)
0.8	378.50±22.49(7)	456.83±129.72(6)	459.25±41.55(4)[f]	495.00±104.59(4)
0.9	348.33±38.25(9)	421.44±39.32(9)	417.60±48.39(5)	379.83±28.08(6)
1.0	306.70±36.69(10)	363.67±50.53(9)	451.89±50.41(9)[f]	369.20±25.67(10)
1.2	299.30±16.93(10)	291.22±15.87(9)	336.75±31.71(8)	-----[e]
1.3	-----[e]	286.50±24.37(10)	337.70±43.57(10)	-----[e]

[a] Male mice were administered 25 ug/kg HC-3 intraperitoneally 30 minutes before, concurrently or 2 minutes after increasing doses of physostigmine (ip).

[b] Time to death is expressed as mean ± S.E.M. in seconds.

[c] Number in parenthesis represents number of deaths per group (n=10).

[d] α=all mice survived.

[e] (-)=not measured, since LD_{100} reached at a lower dose.

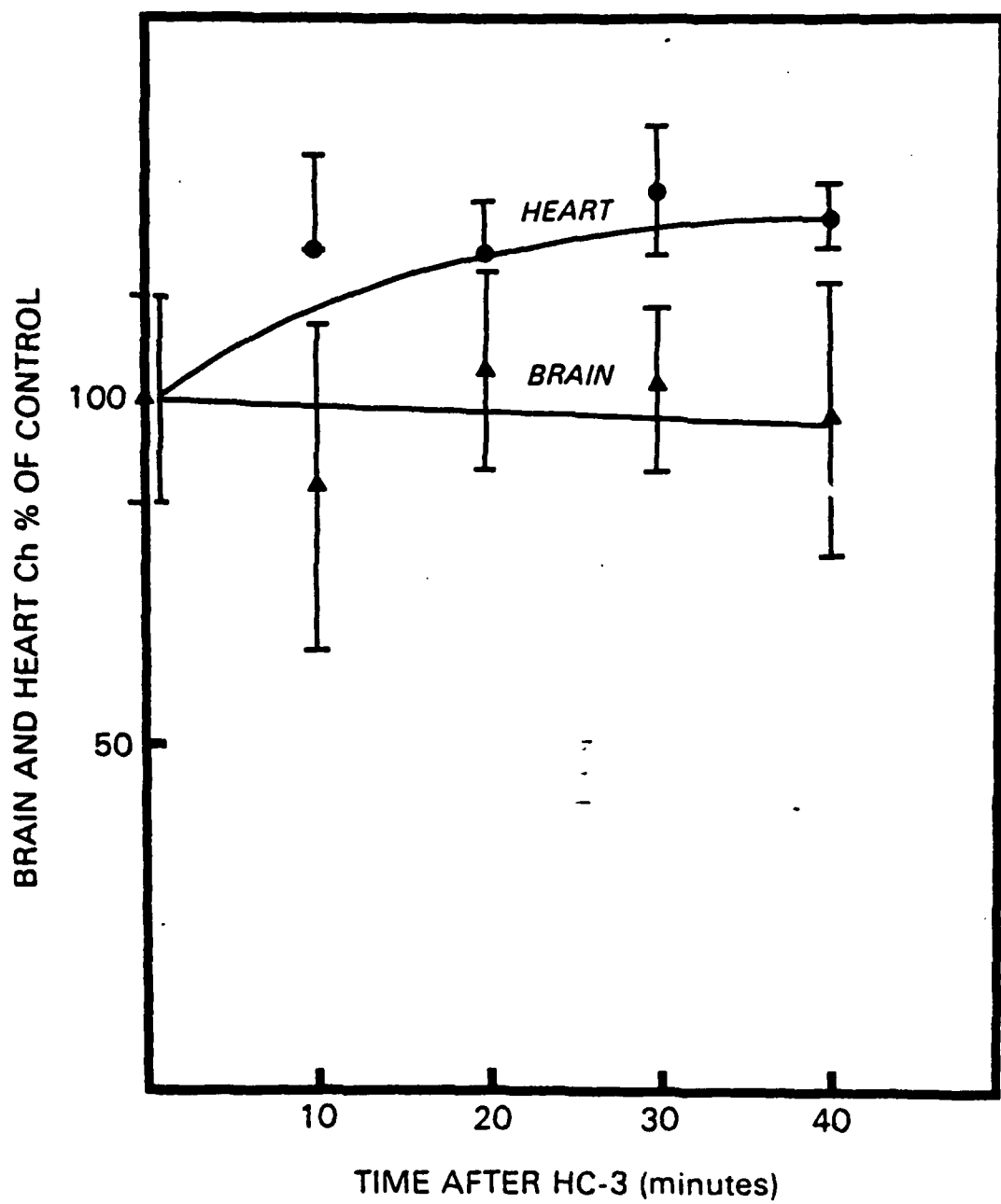[f] 0.025, compared to physostigmine alone.

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TABLE 2

Effect of Hemicholinium-3 (HC-3)[a] on the Time to Death[b] of Mice

Treated with Neostigmine

Dose Neostigmine mg/kg	HC-3 Pretreatment Neostigmine	HC-3 Neostigmine Concurrently	Neostigmine HC-3 Postreatment
0.3	348(1)[c]	677(1)	α[d]	α
0.4	282.60±56.35(5)	616(1)	α	α
0.5	673.00±453.47(3)	510.00±265.87(2)	607.50±203.54(4)	α
0.6	479.83±73.56(6)	299.00±24.04(2)	661(1)	519.33±91.65(3)
0.7	442.00±52.01(8)	570.00±231.28(3)	576.50±83.51(6)	429.17±102.81(6)
0.8	410.44±51.12(9)	601.13±76.99(8)	667.11±98.66(9)	651.33±187.24(3)
0.9	479.80±61.01(10)	389.25±447.72(8)	786.80±208.94(5)	415.25±84.66(4)
1.0	-----[e]	583.10±71.33(10)	623.10±103.71(10)	418.43±83.20(7)
1.1	-----	-----	-----	363.60±63.24(10)

[a]Male mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes
 before, concurrently or 2 minutes after increasing doses of neostigmine (ip).
[b]Time to death is expressed as mean ± S.E.M. in seconds.
[c]Number in parenthesis represents number of deaths per group (n=10).
[d]α=all mice survived.
[e](-)=not measured, since LD_{100} reached at a lower dose.

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TABLE 3

Effect of Intraperitoneal Hemicholinium-3 (HC-3)[a] and Physostigmine (LD_{50}) on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Brain

	Control	Physostigmine (0.69 mg/kg)[i]	HC-3 Pretreatment + Physostigmine (0.72 mg/kg)[i]	HC-3 Physostigmine Concurrently (0.83 mg/kg)[i]	Physostigmine + HC-3 Post-treatment (0.85 mg/kg)[i]
ACh[b] nmole/g	30.29 ±2.40	43.14[c] ±5.23	44.08[d] ±3.12	58.66[e,g] ±2.81	50.53[e] ±2.90
Cb[b] nmole/g	22.16 ±2.45	32.33[c] ±3.45	32.99[f] ±1.87	47.91[e,h] ±3.04	38.17[e] ±2.70

[a] Male mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD_{50} doses of physostigmine (ip).

[b] Values are expressed as mean ± S.E.M. n=8-15.

[c] $p<0.05$, [d] $p<0.01$, [e] $p<0.001$, [f] $p<0.025$, compared to control.

[g] $p<0.01$, [h] $p<0.001$, compared to physostigmine alone.

[i] The LD_{50} for physostigmine is shown in parenthesis as taken from fig. 2.

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TABLE 4

Effect of Intraperitoneal Hemicholinium-3 (HC-3)[a] and Physostigmine (LD_{50}) on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Heart

	Control	Physostigmine (0.69 mg/kg)[g]	HC-3 Pretreatment + Physostigmine (0.72 mg/kg)[g]	HC-3 Physostigmine Concurrently (0.83 mg/kg)[g]	Physostigmine + HC-3 Post-treatment (0.85 mg/kg)[g]
ACh[b] nmole/g	3.03 ±0.45	3.35 ±0.60	3.10 ±0.37	4.34 ±0.75	4.22 ±0.45
Cb[b] nmole/g	38.80 ±3.53	53.40[c] ±5.72	87.26[d,e] ±4.78	73.75[d,f] ±3.73	63.66[d] ±4.55

[a]Male mice were administered 25 ug/kg HC-3 intraperitoneally Iip) 30 minutes before, concurrently, or 2 minutes after LD_{50} doses of physostigmine (ip).

[b]Values are expressed as mean ± S.E.M. n=8-15.

[c]$p < 0.05$, [d]$p < 0.001$, compared to control.

[e]$p < 0.001$, [f]$p < 0.005$, compared to physostigmine alone.

[g]The LD_{50} for physostigmine is shown in parenthesis as taken from fig. 2.

TABLE 5

Effect of Intraperitoneal Hemicholinium-3 (HC-3)[a] and Neostigmine (LD_{50}) on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Brain

	Control	Neostigmine (0.55 mg/kg)[i]	HC-3 Pretreatment + Neostigmine (0.72 mg/kg)[i]	HC-3 Neostigmine Concurrently (0.70 mg/kg)[i]	Neostigmine + HC-3 Post-treatment (0.80 mg/kg)[i]
ACh[b] nmole/g	30.29 ±2.40	41.48[c] ±1.76	33.98[f] ±2.26	43.75[e] ±2.26	34.72[f] ±2.05
Cb[b] nmole/g	22.16 ±2.45	39.17[d] ±1.48	53.38[d,g] ±2.55	48.86[d,g] ±2.15	46.98[n] ±1.44

[a] Male mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD_{50} doses of neostigmine (ip).

[b] Values are expressed as mean ± S.E.M. n=8-15.

[c] $p<0.01$, [d] $p<0.001$, [e] $p<0.005$, compared to control.

[f] $p<0.025$, [g] $p<0.001$, [h] $p<0.01$, compared to neostigmine alone.

[i] The LD_{50} for neostigmine is shown in parenthesis as taken from fig. 5.

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TABLE 6

Effect of Intraperitoneal Hemicholinium-3 (HC-3)[a] and Neostigmine (LD_{50})
on the Acetylation (ACh) and Choline (Ch) Concentration of Mouse Heart

	Control	Neostigmine	HC-3 Pretreatment + Neostigmine	HC-3 Neostigmine Concurrently	Neostigmine + HC-3 Post-treatment
		$(0.55 \text{ mg/kg})^i$	$(0.72 \text{ mg/kg})^i$	$(0.70 \text{ mg/kg})^i$	$(0.80 \text{ mg/kg})^i$
ACh[b] nmole/g	3.03 ±0.45	5.64[c] ±0.83	5.21[d] ±0.57	3.02[h] ±0.41	4.46 ±0.69
Cb[b] nmole/g	38.80 ±3.53	38.07 ±3.42	75.23[e,g] ±4.51	55.79[f,g] ±2.08	58.45[f,g] ±4.21

[a] Male mice were administered 25 ug/kg HC-3 intraperitoneally (ip) 30 minutes before, concurrently, or 2 minutes after LD_{50} doses of neostigmine (ip).
[b] Values are expressed as mean ± S.E.M. n=8-15.
[c] $p<0.05$, [d] $p<0.025$, [e] $p<0.001$, [f] $p<0.01$, compared to control.
[g] $p<0.001$, [h] $p<0.05$, compared to neostigmine alone.
[i] The LD_{50} for neostigmine is shown in parenthesis as taken from fig. 5.

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DISCUSSION

This study was based on the hypothesis that drugs which inhibit the uptake of
choline will antagonize the toxicity of acetylcholinesterase inhibitors.
Hemicholinium-3 (HC-3) antagonized the toxic-lethality of physostigmine and
neostigmine by shifting the lethal dose-response curve to the right, increasing
the LD50 and increasing the time to death. The decrease in toxicity was not
always associated with a decrease in acetylcholine (ACh) levels. Therefore,
the hypothesis proved correct but the mechanism remains unclear.

Pretreatment (30 minutes) with HC-3 should be the most effective treatment
schedule since HC-3 is a well known inhibitor of choline uptake into the nerve
terminal (14, 15, 19) resulting in a long lasting depletion of acetylcholine
Therefore, concurrent treatment should be less effective and postreatment
(2 minutes) the least effective. However, the present study demonstrated that
concurrent and postreatment are more effective than pretreatment. The effective-
ness of HC-3 in antagonizing the toxicity of physostigmine and neostigmine may not
solely be due to interference with presynaptic choline uptake.

In addition to a presynaptic mechanism of action, HC-3 has also been shown to
possess a postsynaptic mechanism by blocking the nicotinic receptor (46, 47)
Further evidence for a different mechanism of action (other than an effect on
uptake) was provided by the present combination studies involving acetylcholinesterase
inhibitors and hemicholinium-3 (Tables 1-4). Since the dose of the cholinesterase
inhibitor represented the LD50 dose taken from the dose-response curve, the

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acetylcholine levels listed in table 3-6 may be related to the cause of death in mice. Hemicholinium-3 (HC-3) had variable effects on acetylcholine levels in brain and heart when combined with physostigmine and neostigmine. As expected, physostigmine alone caused an increase in acetylcholine (ACh) concentration in the brain and HC-3 did not reduce the increase in ACh concentration (Table 1). This would be anticipated since HC-3 has been shown not to cross the blood brain barrier (13, 32, 34). Physostigmine increased the level of acetylcholine proportional to the LD50.

Since cholinesterase inhibitors probably exert their toxicity by a combination of central and peripheral mechanisms, the effect of physostigmine and HC-3 was also studied in the heart. Physostigmine alone did not cause a significant increase in the concentration of heart acetylcholine, nor did HC-3 cause a reduction in the level of acetylcholine compared to control. Dieterich et al. (48), using an in vitro heart preparation demonstrated that a 10^{-4}M concentration of physostigmine had no effect on acetylcholine levels while a lesser 10^{-7} M concentration did cause an increase. These authors speculated that at the higher concentration (i.e. toxic doses in our experiment), physostigmine prevented the formation of "surplus" acetylcholine pools. Thus the data in table 2 may also be evidence for this paradoxical effect.

Unlike physostigmine, neostigmine alone did cause an increase in the level of acetylcholine in the heart. Concurrent HC-3 treatment and postreatment did appear to reduce the increase in acetylcholine caused by neostigmine. However, this may be due more to the variablility of the effect of neostigmine on the

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heart than an effect of HC-3, since HC-3 alone had no effect on the heart
(Fig. 7). Also, concurrent and postreatment had a greater effect than pretreatment
which would not be the expected result. It is also possible that the heart does
not reflect the toxicity of cholinesterase inhibitors as well as diaphragm.

Neostigmine was selected as a peripheral control for physostigmine. Surprisingly,
neostigmine alone caused an increase in acetylcholine levels in the brain following
systemic administration. The consensus in the literature has shown that
neostigmine does not get into the brain following systemic administration (49)
There are, however, a few indications that neostigmine may cross the blood-brain
barrier (50-52). The present study confirms and expands the previous studies and
provides further evidence that neostigmine does cross the blood-brain barrier. In
addition to demonstrating a significant effect on acetylcholine and choline in the
brain (Table 3), neostigmine was also shown to have a significant effect on
inhibiting acetylcholinesterase in the brain following systemic administration.
Therefore, in high doses neostigmine does cross the blood-brain barrier.

Neostigmine and physostigmine had significant effects of increasing brain and
heart choline (Table 3-6). This effect is apparently not due to HC-3 since
HC-3 alone did not affect brain or heart choline significantly (figure 8).
Karlen et al. (53) also observed increased levels of choline in the mouse
brain as a result of physostigmine. These authors speculated that physostigmine.
may impair the efflux rate of choline from the brain. However, the present
study has demonstrated that acetylcholinesterase inhibitors also increase
choline levels in the heart. The mechanism for this observed increase in

choline production is not known. Any alteration must be a result of a change in either the generation of efflux of choline. Since only physostigmine, not neostigmine, has been shown to inhibit the release of acetylcholine (4), it is unlikely that this is a satisfactory explanation for the observed actions.

The failure of hemicholinium-3 (HC-3) to have the desired effect on diisopropyl-fluorophosphate (DFP) toxicity is unexplained (Fig. 7 and 8). In fact, various treatments with hemicholinium-3 actually increased the toxicity. The purity of the DFP was questionable even though two suppliers and several batches were used. The LD50 (10 mg/kg) never did equal the LD50 reported in the literature, but this does not adequately explain the discrepancy in the data between the reversible inhibitors and irreversible inhibitors.

In conclusion, HC-3 has been shown to antagonize the toxicity of reversible, but not irreversible acetylcholinesterase inhibitors with variable effects on acetylcholine levels. Data suggests that the mechanism of action of hemicholinium-3 may be related more to a post-synaptic rather than to a pre-synaptic effect. The discrepancy between the effect of hemicholinium-3 on the reversible and irreversible inhibitors remains unexplained.

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